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STUDIES ON THE GONOCOCCUS, III*

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AUTOLYSIS

In previous papers I have called attention to lysis in the gonococcus and have emphasized this phenomenon as being invariably present to a greater or less degree in artificial culture. This study was undertaken with the objects of ascertaining which factors bring about and influence the lytic changes—whether the process is due wholly to the action of enzymes, as is commonly maintained, or to other causes—and of obtaining a more intimate knowledge of the biochemistry of the organisms. This has seemed to me important in view of the practical value such observations might have in the problem of immunity. The material consisted of twenty-two strains of gonococcus isolated by me within the past year from cases of gonorrhea in men and young girls, in addition to several, including six strains of meningococcus, kindly given me by other workers.

The question first to be considered was whether lysis might be due to conditions in the culture media, or to causes within the organisms themselves. Accordingly the strains were grown upon all media specially recommended for cultivating the gonococcus, with the result that all strains showed lysis upon all media,[1] varying only in degree under conditions to be mentioned later. This fact being determined there remained to be shown what element of the media, common to all, initiated the lysis, on the assumption that the conditions of environment were alone responsible.

In order to avoid a multiplicity of media a standard was adopted consisting of salt-free veal broth containing 2 percent agar, neutralized to phenolphthalein, to which was added 5 percent defibrinated rabbit blood.

The optimum reaction was observed by varying from the neutral point. The results showed that while the organisms could be brought to endure considerable variation in reaction, the best condition for all strains was at the neutral point, altho at all points lysis occurred.

The quantity and quality of nitrogen were next investigated. The quantity of nitrogen in the medium was reduced first by eliminating the blood, the

* Received for publication March 19, 1915.

1. Jour. Infect. Dis., 1913, 13, p. 124.

strains being grown upon salt-free, neutral veal peptone agar. This medium gave satisfaction for the propagation of the strains but permitted lysis to occur. The peptone was then omitted from the medium with the same results. This medium has been used for some time by Park and Williams. I have found it serviceable for all strains after the primary culture. While the nitrogen content of this medium is probably complex, it is extremely simple in comparison with the elaborate supply and character in the standard. From this point in the study all transplants were made from this medium. The time limit for observations on lysis was twenty-four hours. Cultures older than one day contain many dead organisms.

The veal was next replaced by nitrogen-bearing salts covering a wide range. Such substances as asparagin and simpler salts, such as potassium nitrate and sodium nitrite, were used successfully in propagation, all cultures being carried through eleven transplantations at intervals of forty-eight to seventy-two hours. It was found that upon such extremely simple media as 2.25 percent agar in distilled water, containing no other substances than small amounts of sodium hydrate or sodium carbonate, the organisms found sufficient nitrogen to maintain existence, but in every case, save as shown later, lysis was noted. Evidently, lysis is not influenced by the amount or character of the nitrogen in the medium.

Coincidently with the nitrogen examination, the veal agar cultures and those of simpler nature were subjected to various degrees of diminished oxygen. Novy jars were used entirely and the oxygen lowered with pyrogallol-sodium hydrate. All cultures, even on the simple media mentioned, were gradually accommodated to full anaerobiosis (10 gm. pyrogallol and 50 c.c. of 25 percent, sodium hydrate solution per liter of space), and continued in this environment for the full eleven generations. Under these unusual conditions the cultures showed no variation in lysis or microscopical appearance from the standard cultures, whereas the gross appearance changed considerably. On the simplest media the growth became very delicate and vapor like, the colonies being visible only with a hand lens and never exceeding a diameter of 0.5 mm. From these cultures transplantation back on the standard medium was made whereupon they resumed the ordinary character of growth. It was also found that variations of temperature within limits of viability produced no change in lysis.

It will be observed that this range of investigation has excluded any possible influences of sugar and fat upon lysis and there remains therefore but one factor in the media for consideration, namely, water. Previous experiences with fluid media had shown me that in them lysis appeared to be greatest. In working with solid media also it was noticed that growth in the more moist regions of the media always showed a greater degree of lysis. This was apparent also in smear preparations for microscopical examination when the substance was taken from the moist portions of the plate or near the water of condensation in the tube. Slight variations in detail of making smear preparations create great changes in microscopical appearance. A simple experiment will illustrate. Remove a trace of material from the edge of a twenty-four-hour colony of gonococcus or meningococcus, smear it quickly upon a slide with a very minute drop of water or salt solution, and dry immediately with an air blast or by blowing sharply on the slide. Upon the same slide make smears in one loop of water, then in two loops, then in three or more pooled, and allow to dry in the air. After fixing and staining with methylene blue or, better, dilute carbol-fuchsin, examination in series will show the extraordinary changes which excess of water will produce in a very short time. The alterations in morphology and

other changes indicative of lysis are too well known to require detailed mention. Briefly, the process begins with increase in size of the coccus and is followed by distortion into a great variety of forms, together with granulation, accompanied from the beginning by diminished brilliancy of staining, extending from blurring to the faintest shadows.

The gonococci may derive excess of water while on a medium from sources other than the medium, as for instance from a very moist atmosphere, from water of condensation, including that which occurs incident to the removal of glassware from incubator temperature into that of the room. The effect of the latter may be shown by placing plates bottom up or agar slants culture surface uppermost after removal from the warm room, when the vapor will form on the growth instead of on the glass. In a word excess of water causes lysis. It will be objected at once that the contention will not hold for the reason that gonococcus and meningococcus will develop in fluid media, and also by reason of the observations of other workers that this group of organisms is very sensitive to lack of moisture.[2] The first objection is admitted. Very many organisms may be induced to propagate upon media ordinarily harmful, and the gonococcus-meningococcus group is no exception. However, my observations have shown that nearly all pure strains avoid the fluid as far as possible by building their growths on the surface and that all cultures in fluid media show extreme lysis, large, poorly staining cocci carrying their maximum load of water, and that transplants from the body of the fluid, which is usually clear when strains are uncontaminated, or from the bottom of the culture tube, are successful only very rarely, probably when fresh particles from the surface growth are settling or have recently reached the bottom.[2] (In defibrinated human blood the cocci may live for a long time). It is admitted also that these cocci adapt their existence to considerable degrees of moisture on solid media with the result that often more luxuriant growth is so obtained, as tho existence depended on rapidity of vegetation. I believe, however, that these exceptions merely prove the rule, for my experience has been that such cultures always show great lysis and are very prone to perish overnight. When in this "water logged" state, the cocci are very sensitive to injury so that rough handling often kills a transplant.

With a view to determining whether an optimum of water would permit growths of this group free from lysis on the medium, a long series of observations was made with many media in varying degrees of dryness. To avoid details the results may be summarized by stating that all strains were cultivated with greater surety and uniformity and with least lysis upon media containing a minimum of moisture. Success with the simple media mentioned, with and without oxygen, depended on attention to this detail. I now use but two media for routine cultures and for isolation, one the salt-free neutral veal 2.25 percent agar, and the other the same to which while at 60 C. is added 2.25 percent defibrinated rabbit blood. Primary cultures are made in the latter and are transferred at once to the former. Before use the

2. Irons: Forscheimer—Billings, Therapeusis of Internal Diseases, 1914, 5, p. 599. Ohlmacher: Jour. Am. Med. Assn., 1915, 64, p. 585.

tubes are dried in desiccators over sulphuric acid or in a dry room at 37 C. until all water of condensation has gone and a point is reached where examination of the surface of the medium with a hand lens shows a slight roughening or puckering. If the agar has cracked, no harm is done. From such dry media the organisms get all the water they require and upon these media a twenty-four-hour growth shows vegetation without lysis. I have found that by the use of the dry blood medium the chief difficulty in securing primary cultures has been overcome. A minimum amount of pus gently distributed over the entire area insures colony formation, whereas on more moist media very often the culture fails. As regards the maintenance of growth, transplants may be made once a week, altho cultures frequently survive for many months, enduring until the medium has dried down nearly to the glass. In such growths secondary colonies or masses appear on the surface of the early growth, spread irregularly, and form cones which slowly flatten out and afford a base for other cones. In this way elevations of 3-5 mm. are built up, from the summits of which successful subcultures may be made. In transplanting, some idea of the requisite dryness of the medium may be had by observing that the material adheres to the surface wherever it comes in contact. If the surface is too moist the material, like fat, slips over it and rolls up into ridges.

Lysis in suspension in water, salt solution, or various other solutions, depends for the most part on two factors: the concentration of the suspension and the ionic content of the solution. In salt solution, lysis occurs in all concentrations, becoming progressively less up to 25 percent, beyond which a slower disintegration occurs. Sodium fluorid checks lysis in about 10 percent solution. Flocculation is readily produced by salt solutions at 0.9 percent and above, by solutions of the heavy metals, by acid ions, and in nearly all dilutions by the salts of the alkaline earths. Compact flocculation as with $CaCl_2$ delays lysis, while slight flocculation as with normal salt solution tends rather to increase it. The hydrosol colloids permit lysis apparently in direct proportion to the available water. Suspension colloids such as serum and lecithin-water favor lysis.

Those agents which prevent lysis have been well reviewed in recent literature. Many dehydrating substances such as 50-95 percent alcohol (McClintock and Clark), 25-50 percent acetone, strong solutions of

sugar (syrup), preserve the cocci for considerable periods of time. These substances extract certain ingredients from the organisms, especially alcohol, the effect of which will be mentioned later. While weak solutions of the alkali oleates do not prevent lysis, stronger solutions prevent it. Fatty substances, such as neutral fat, olive oil, lanolin, certain cosmetics, hydrocarbons like vaselin, and all substances more or less free from water, prevent lysis. From the surface of lanolin whereon small amounts of culture material had been placed, I have, after a month, obtained microscopical preparations showing the cocci to be in as perfect condition as to shape, size, and staining reactions as if taken fresh from culture. A point in this connection of greater importance is the fact that the viability of gonococci may be maintained in such substances for days. Small loops of gonococcus culture were planted upon lanolin sterilized and slanted in tubes. After five days at room temperature successful cultures were obtained on the standard medium; also from surface and buried inoculations on "cold cream," cultures were recovered on the fifth day. Plants on vaselin (Kahlbaum's white) at 20 C. and at 4 C. have yielded cultures after forty-eight hours. The behavior of culture material on such substances depended on the degree of moisture present in the culture. Wet material, after planting, gradually ran together into small, colony-like masses, while dry, somewhat granular material remained as placed. Such masses are easily distinguished from droplets of condensation. In culturing from such substances there were many failures because of the difficulty in freeing the organisms from the fatty envelope.

Glycerol 75-100 percent prevents lysis and preserves the cocci best of all substances examined. The appearances remain unchanged after months in this menstruum. Lysis occurs in 50 percent glycerol. The behavior of gonococcus and meningococcus, especially the former, in suspension in glycerol is peculiar. Small loops of culture material were suspended in as fine a state of division as possible at the surface of sterile pure glycerol in tubes kept at 4 C. and at 20 C. After two or three days the particles near the surface were evenly distributed in fine suspension, below which was a layer of coarser granules out of which floccules of larger size had separated and were settling, leaving delicate, white streamers in their track. These white, granular looking floccules appeared to increase in size as they settled in the

fluid. Microscopic examination showed them to consist of masses of cocci of perfect shape and stain-power, apparently undergoing growth. Individual floccules, pipetted into fresh glycerol, repeated the process on a smaller scale. Floccules from gonococcus transferred to dry blood agar from glycerol tubes kept at 4 C. for seventy-two hours, yielded abundant colonies, and scattered colonies were obtained from tubes kept at 20 C. for six days. The cocci are extremely sensitive to injury in glycerol, as may be seen in microscopical preparations that are made at all roughly. The addition of 1 percent boric acid to glycerol made no difference in viability of the cocci. Cultures of the meningococcus were not made. A small drop of glycerol containing a gonococcus floccule from a tube kept at 20 C. for forty-eight hours was spread over a small area on the abductor surface of the last phalanx of the little finger. From this an impression was made after one hour on dry blood agar. Incubation for forty-eight hours yielded a single gonococcus colony from which sub-cultures were made. Successful cultures were never obtained from glycerol kept at 37 C. after twenty-four hours. The dry blood agar absorbs considerable amounts of glycerol in a short time. All these observations lead to the conclusion that lysis of this group of organisms is initiated by water in excess.

The next inquiry concerned the nature of the changes in the gonococcus induced by water, and to this end it was necessary to examine the normal organic substances in the organisms. My attention was drawn first to the fats by several observations, namely: the penetrating and characteristic odor of some samples of freshly dried gonococcus substance, the relation existing between the gram character of many bacteria and their lipoid content,[3] the fact that gonococcus suspensions and autolysates showed on shaking a more or less permanent foam due partly to proteid and partly to soaps, and the presence in salt solution autolysates and suspensions of a lipase which produced acid from neutral fat (olein).

QUALITIVE EXAMINATION OF THE FATS

Large 24-36 hour cultures of gonococcus grown on fat-free medium (slightly acid veal broth extracted with ether, neutralized to phenolphthalein 2.25 percent agar) were saponified and the solution after acidifying was distilled in steam. The early fractions of the distillate showed fine, oily drops on a clear fluid, later fractions gave a slightly cloudy distillate carrying abundant large, white, floating flakes, considerable quantities of which accumulated in the condenser, while the last portions to distill over were clear.

3. Tamura: Ztschr. f. Physiol. Chem., 1914, 89, p. 304.

The entire distillate, after the addition of a slight excess of alkali, was evaporated to dryness on a bath and the residue taken up in a small quantity of 20 percent sulphuric acid. Certain higher fatty acids appeared on the surface as oily drops and solid flakes. By siphonage the acid solution was separated and, on salting, showed still lower volatile fatty acids, and extraction of the salted solution with ether yielded appreciable traces of others.

The non-volatile acids remaining after distillation were then recovered by extraction with ether. At 20 C. these were solid, yellowish substances with the same pungent odor as that of the dry substance.

By the lead-salt-ether method the unsaturated fluid fatty acids were separated. These were dark red in color, with slight odor. The remaining saturated fatty acids were solid, rather bulky, and white.

I have been unable to identify these acids. Certain of the volatile acids solidify from etheral solution at 12 to 13 C. and melt at 6 to 5 C. The melting point of the non-volatile saturated acids could not be determined exactly, 43 to 45 C. being the figures oftenest obtained.

To summarize, the gonococcus yields considerable quantities of rather bulky fatty acids, volatile and soluble in water and salt solution, non-volatile, saturated and unsaturated.

QUANTITATIVE ESTIMATION OF THE FATS

These estimations have yielded amounts which varied considerably with the physical state of the culture material and the method of treatment. Washing and drying produce great changes, as do also the age of the culture and the amount of moisture it contains. Accordingly in each estimation the state of the substance is noted.

Twenty-four-hour mass cultures of gonococcus grown on fat-free medium were washed repeatedly with water, then dried in vacuo over P_2O_5 and powdered. By saponification method, the following results were obtained:

Weight of Substance	Volatile Acids	Unsaturated Acids	Saturated Acid
2.0140	0.0279	0.0067	0.0549

This analysis was selected from many as representing an average of fatty acids yielded by substance prepared in this way. As will appear later, these figures are too low, especially for the volatile and unsaturated fatty acids. In using smaller quantities of substance desiccation by alcohol was tried, but abandoned when it was found that alcohol removed rather large amounts of fat.

In order to determine approximately in what condition the fats exist in the unaltered gonococcus, a thirty-six-hour culture was washed once in water, centrifugated at 6,000 and the sediment shaken out with petroleum ether, 50 percent alcohol, the solutions separated, the ether solution shaken with fresh 50 percent alcohol, and vice versa, several times, and the appropriate solutions added together. At the same time a washed and dried specimen was examined by the same method. The results follow:

Weight of Substance	Neutral Fats	Fatty Acids and Soaps
Wet 0.6850	0.0133	0.005
Dry 1.8651	0.054	0.003

The water in which the fresh culture had been washed yielded fatty acids 0.0076.

A control estimation shows that not all the fats are obtained by such a method. Air-dried powdered gonococcus substance was extracted with petroleum ether in the cold by long grinding and shaking. After separation the ether was treated as above. The residue was then extracted with fresh petroleum ether, on a water bath, with a condenser, for two hours and the ether again separated. The residue was finally saponified. The results were:

Weight of Substance	First Filtrate	Second Filtrate	Residue
2.8464	Fats 0.0636	0.0196	.....
	Acids 0.0047	.....	0.008

To determine the amount of fat liberated by the gonococcus on suspension in water, the following experiment was made. The growth of thirty-six-hour cultures was received into distilled water and divided into equal portions. One portion stood at 20 C. for two hours and the other was heated at 65 C. for the same length of time. Both portions were then centrifugalized at high speed until sedimentation was complete. Fluids and sediments were then examined. As control, a quantity of culture equal to each portion was quickly removed into alcoholic potash, saponified and estimated, with results as follows:

Temperature	Volatile Fatty Acids	Non-Volatile Fatty Acids
20 C. Fluid	0.0072	0.0018
Sediment	0.0244	0.0114
65 C. Fluid	0.0216	0.0036
Sediment	0.0156	0.0078
Controls 1.....	0.0238	0.0174
2.....	0.0299	0.0403

The amount of non-volatile acids obtained for Control 2 was obtained by raising the boiling point of the acidified solution and adding a trace of $CuSo_4$ as catalyst. This shows that a considerable quantity of fat is not readily dissociated by ordinary means and that the figures before given for the non-volatile fatty acids are too low. Further work has led me to believe that at least a part of the increased yield is due to unsaturated fatty acids in a lipoid combination which is not hydrolysed until the nitrogen is thoroughly broken up.

The following table indicates that the fats of the gonococcus probably differ from the fats of other organisms in character and proportion. Cultures from equal surface areas were used:

Organism	Volatile Acids	Unsaturated Acids	Saturated Acids
Staphylococcus	None	0.0032	0.0016
Anthrax	None	0.0146	0.0086
Typhoid	0.008	0.0049	0.0144

To obtain an idea of the proportion of fatty substances of the gonococcus extractable by ether and alcohol and the relation of these substances to the nitrogen, the following analyses were made. In testing small quantities of material wherein the nitrogen content is expressed in milligrams, adaptations of the micro methods of Folin were found serviceable.

A quantity of air-dried gonococcus substance, finely powdered, was extracted with a large volume of ether in the cold, with frequent shaking for twenty-four hours. The clear, colorless extract yielded on evaporation a white, fat-like body. Re-dissolved in ether, the solution gave a faint cloud with platinic chlorid but none with acetone. The gonococcus residue, after ether extraction, was then extracted with 99 percent alcohol at room temperature, with frequent changes, for seventy-two hours. The extract was clear, slightly yellow, and deposited a reddish residue soluble in absolute alcohol. The solution gave a white cloud with water and with acetone, and an abundant yellow precipitate with platinic chlorid.

The gonococcus residue after alcohol extraction was then saponified and the remaining fats recovered as fatty acids.

CONTROLS

0.2002 air-dried gonococcus gave 0.0256 total nitrogen (Folin).
0.35 air-dried gonococcus gave 0.0415 total nitrogen (Kjeldahl).
0.501 air-dried gonococcus gave 0.0647 total nitrogen (Folin).
0.501 air-dried gonococcus gave 0.0261 fatty acids.

The calculated total nitrogen in 3.0105 gonococcal substance was 0.3853, the calculated fatty acids 0.15. Quantity of alcoholic extract, 130 c.c.; total nitrogen in 10 c.c. of extract, 0.000197; calculated total nitrogen in extract, 0.002575. Total fatty acids in 10 c.c. extract, 0.0023; calculated fatty acids in extract, 0.03. Ether extract gave 0.0001 total nitrogen and 0.0184 fatty acids. Residue by saponification gave fatty acids, 0.0279.

There is a discrepancy in the fats which is not explained.

A second record is submitted of a similar analysis save that ether extraction was omitted.

Calculated total nitrogen in 3.1975 gonococcal substance, 0.4093; fatty acids 0.166. Quantity of alcoholic extract, 130 c.c.; determined nitrogen in 10 c.c. extract, 0.0002; calculated nitrogen in 10 c.c. extract, 0.002624. Determined fatty acids, 0.005; calculated fatty acids, 0.065. Residue by saponification, 0.068.

From these analyses it is observed that extraction of dried gonococcus substance with ether in this manner yields a small proportion of fatty substance containing a trace of nitrogen, while alcoholic extracts yield about one-half the total fats containing less than 1 percent of the total nitrogen. The inference is that some of the nitrogen is closely bound to the fat in the gonococcus.

Having observed the extent to which lysis of gonococcus in water suspension liberated fats, I deemed it advisable to ascertain to what extent and in what form nitrogen is set free. If lysis were due to enzyme activity, the nitrogen would probably be, at least in part, present as ammonia. Accordingly many tests were made but it was found that little information could be gained by estimating NH_3. All suspensions and autolysates showed NH_3 in measurable amounts which apparently increased with the time, at moderate temperatures, and diminished at high temperatures. Of the many suspensions examined those heated to 58 C. for thirty minutes, then kept at 37 C. for forty-eight to seventy-two hours showed the largest quantities of nitrogen as NH_3 in the autolysates.

LYSIS IN RELATION TO TOXICITY AND ANAPHYLAXIS

In order to observe the degrees of lysis in sera and the property of suspensions of gonococcus undergoing lysis for the production of anaphylatoxin in sera, the following experiment was made.

The cultures used were dry, twenty-four-hour agar-grown, and to avoid previous lysis were emulsified directly in the sera. The serum suspensions contained no agar. After incubation at 37 C. the suspensions were centrifugalized until perfectly clear of cocci (4,000 revolutions for 20 to 30 minutes) and injected intravenously into guinea-pigs, weighing 225 to 250 gm. each.

TABLE 1.

THE EFFECTS OF INJECTION OF GONOCOCCI IN VARIOUS SERA

Guinea-Pig	Quantity of Culture	Serum	Hours at 37 C.	Amount Injected	Results
1	0.5 slant	Normal rabbit	1.5	4 c.c.	Mild shock, ill later, died 12 hours Autopsy negative
2	0.5 slant	Inactivated rabbit	1.5	4 c.c.	No shock, ill later, lived
3	0.5 slant	Immune rabbit (old)	1.5	4 c.c.	Itching, ill later, lived
4	0.25 slant	Immune rabbit, reactivated	1.5	4 c.c.	Severe shock, ill later, died 10 hours
5	0.25 slant	Normal guinea-pig	1.5	4 c.c.	Slight shock, ill later, died 8 hours
6	0.25 slant	Immune goat (old), react.	1.5	4 c.c.	Slight shock, ill later, lived
7	0.25 slant	Normal guinea-pig	0.5	4 c.c.	No shock, ill later, lived
8	1 slant	Normal guinea-pig	3	4 c.c.	Severe shock, ill later, died 8 hours
9	1 slant	Normal guinea-pig	4	4 c.c.	Death 2 minutes; typical anaphylaxis with emphysema, blood fluid
10	1 slant	Normal guinea-pig	4	4 c.c.	Death 2.05 minutes; anaphylaxis
Control	.....	Normal rabbit	4	4 c.c.	No effect
Control	.....	Normal guinea-pig	4	4 c.c.	No effect

The next experiment was made to determine whether the late death of the animals was due to poisonous substances from the gonococcus alone. The suspensions in water and salt solution were, as in the preceding, uncontaminated and were treated in the same manner as the serum suspensions. The injections of 4 c.c. were given intravenously into guinea-pigs of the same average weight. Salt solution was used by preference to facilitate centrifugalization.

From this experiment it was concluded that the late death of the animals was probably due to substances derived from lysis of the gonococcus.

Table 3 shows approximately the nitrogen values of such suspensions and autolysates as were used in the preceding experiment, together with others subjected to wider ranges of temperature. The same quantities of material were employed and the emulsions prepared in the same manner. The results are expressed in fractions of a milligram.

The results embodied in the tables, together with many control and duplicate experiments for which records are not given, show several points of interest.

It is possible to induce anaphylaxis easily in guinea-pigs with gonococcus suspended in homologous serum. Rabbit serum does not answer so well because of the large quantities of material necessary, and human serum is too toxic for guinea-pigs to be serviceable.

TABLE 2

THE EFFECTS OF INJECTION OF GONOCOCCI IN WATER AND SALT SOLUTION

Guinea-Pig	Culture	Fluid	Temperature	Time	Results
11	1 slant	Distilled water then salt to 0.9	37 C.	4 hours	Paralysis, temporary; quick recovery, ill, died 24 hours
12	1 slant	Salt solution	37 C.	0.5 hours	Paralysis, temporary; quick recovery, ill, died 10 hours
13	1 slant	Salt solution	37 C.	0.5 hours	Paralysis, temporary; quick recovery, ill, died 8 hours
14	1 slant	Salt solution	37 C.	4 hours	Paralysis, temporary; quick recovery, ill, died 10 hours
15	1 slant	Salt solution	37 C.	6 hours	Paralysis, temporary; quick recovery, ill, died 18 hours
16	1 slant	Salt solution	37 C.	48 hours	No effect, ill later, lived
17	1 slant	Salt solution	58 C.	1 hour	No effect, ill later, lived†
18	1 slant	Salt solution	58 C.	0.25 hour	Paralysis, temporary; ill later, died 18 hours
19	1 slant	Salt solution	58 C.*	.....	Paralysis, temporary; ill later, died 10 hours
20	1 slant	Salt solution	70 C.	1 hour	No effect, ill later, lived
21	1 slant	Salt solution	70 C. then 48 hr.	1 hour 37	No effect, slight illness later, lived
Control	.....	Salt solution	20	.....	No effect
Control	1 slant	Salt solution	20	.....	Paralysis, ill later, died 6 hours

* Centrifugated at once.

† The salt solutions were heated to the temperatures indicated before the addition of the culture.

Fresh autolysates of gonococcus in water, salt solution, and serum are toxic for guinea-pigs and all other animals. Autolysates formed at moderate temperatures within short periods of time are fatal for guinea-pigs. The illness is identical with that observed following intraperitoneal injections of gonococcus suspensions,[4] the invariable feature of which is a rapid fall in temperature.

The toxicity of autolysates apparently does not depend upon the quantity of nitrogen present but probably upon the character of the nitrogen in the early stage and is due to the anaphylatoxin producing power of substances derived from the cocci.[5]

4. Warden: Jour. Infect. Dis., 1913, 12, p. 104.
5. Dold: Bakterien Anaphylatoxin, Jena, 1912.

Lysis occurs in water and in salt solution at all temperatures below the coagulating point of proteid. At low temperatures, lysis is not at once manifest in the suspension but becomes apparent with centrifugation. As more of the proteid is coagulated, or otherwise altered by increasing temperature, the percentage of total nitrogen in the fluid diminishes. The rate and degree of lysis increase with the length of

TABLE 3
THE NITROGEN VALUES OF GONOCOCCAL SUSPENSIONS AND AUTOLYSATES

Culture	Temperature, C.	Time	Nitrogen		
			Amount of Fluid in Milligrams	Amount of Sediment in Milligrams	Amount of Suspension in Milligrams
24-hour dry	4	2 hr.	.....	0.4	0.965
24-hour dry	4	2 hr.	0.55	.....	.....
24-hour dry	20	at once	.....	.....	1.20
24-hour dry	20	20 min.	.....	.....	0.726
48-hour dry	20	20 min.	.....	Trace	0.8
48-hour wet* ...	20	20 min.	1.0	0.4	.....
24-hour dry	20	20 min.	0.36	0.27	.....
24-hour dry	20	2-3-4 hr.	0.55	.....	.....
24-hour dry	20	6 hr.	.....	.....	0.765 amino †
24-hour dry	20	6 hr.	0.37	0.51	0.65
24-hour dry	37	20 min.	.....	.....	.....
24-hour dry	37	20 min.	0.92	0.19	0.714
24-hour dry	37	20 min.	0.44	0.37	.....
24-hour dry	37	1 hr.	1.2	Trace	.....
48-hour wet	37	1 hr.	0.43	0.38	.....
24-hour dry	37	4 hr.	0.37	0.36	.....
24-hour dry	37	72 hr.	0.65	Trace	.....
24-hour dry	58	1 hr.	0.66	Trace	.....
24-hour dry	58	2 hr.	0.44	Trace	.....
24-hour dry	58	4 hr.	0.42	0.30	.....
24-hour dry	70	1 hr. then 20 C.	0.26	0.27	.....
24-hour dry	70	2 hr.	.....	.....	.....
24-hour dry	70	1 hr. then 30 C. 48 hr.	0.17	0.19	.....
24-hour dry	100	1 min. cooled to 20 C.	0.2	0	.....

* Very moist culture, grown on wet medium.
† Van Slyke method.

time of growth and water content of the culture, and vary with the lytic character of the strain.

Old cultures and very wet cultures apparently yield larger amounts of nitrogen to the water in a short time than fresh and dry cultures.

The amounts of nitrogen in fluid and sediment never equal the total nitrogen of the culture material estimated directly.

The total nitrogen of suspensions appears to diminish in amount the longer the suspensions have stood.

Lysis occurs at temperatures that check the activity of enzymes. The degrees of lysis in the individual cocci as observed by microscope in these experiments were so difficult to distinguish that no attempt at classification was made. Lysis was great in most instances and in serum invariably extreme. In general it appeared that extremes of temperature afforded better appearances of preservation.

PREPARATION OF GONOCOCCUS ANTIGEN

The examination of water autolysates as prepared by me for gonococcus complement fixation tests, showed them to contain proportional quantities of the substances already mentioned. The antigens are made in two ways: one by allowing suspensions to stand at 20 C. for two hours, centrifugalizing at high speed, and preserving the fluid sterile; the other by heating the suspension to 58 C. for two hours, centrifugalizing, and heating the fluid at 75 C. for thirty minutes. When freshly prepared, one-half made in the manner first mentioned contained 2.3 mg. total nitrogen, 0.0181 gm. fatty acids and 0.00017 gm. phosphorus as pyrophosphate, while one-half made in the second manner showed little or no variation save in the reduction of the fatty acids about one-half. Examinations of old antigens showed the total nitrogen apparently to have been much reduced in amount, while the fats were correspondingly less. Similar changes have been noted in vaccines.

TOXICITY OF FATS AND EXTRACTS

The following experiment was made to determine whether the alcoholic extract of gonococcus substance and an alcoholic solution of the fatty acids were toxic for guinea-pigs. The solutions used were (1) the alcoholic extract of dried gonococcus known to contain per 10 c.c., 0.005 gm. fatty acids and 0.2 mg. nitrogen, and (2) an alcoholic solution of gonococcus fatty acids known to contain per 1 c.c., 0.005 gm. Varying quantities of the solution were diluted with salt solution in such a manner that after evaporation of the alcohol at 56 C. the total quantity for injection was 4 c.c. The material after thorough shaking varied from a slight milkiness to dense opacity. Injections were intravenous into guinea-pigs weighing 225-250 gm. each.

It appears that the solutions given by this method were not toxic, but that as heavy suspensions they might cause death mechanically, or by inducing toxicity in the serum of the animal.

DISCUSSION

From what is known of the gonococcus it may be safely assumed that the limiting layer is extremely sensitive to changes in surface tension and very permeable by water. The almost naked cell body is easily bruised and altered by physical states which appear to have slight or no effect on many other bacteria. Certain electrolytes, such as NaCl, favor the permeability of the cocci, while others, such as HCl and $CaCl_2$, are more or less antagonistic to it. In a previous paper I stated that HCl prevented lysis. This is true only for a time, as the acid ultimately produces an increase of permeability.[6] The

TABLE 4
THE EFFECT OF INJECTION OF ALCOHOLIC EXTRACTS OF GONOCOCCI

Guinea-pig	Solution	Results
22	No. 1, 1 c.c.	No effect.
23	No. 1, 5 c.c.	Twitching, slight paralysis, quick recovery.
24	No. 1, 10 c.c.	Death 2 min. pulmonary emphysema, blood clotted.
25	No. 2, 1 c.c.	No effect.
26	No. 2, 5 c.c.	No effect, slightly ill later, quick recovery.
27	No. 2, 10 c.c.	Forced respiration, lies on side, recovery in one hour.

substance of the cocci is regarded to be soft and delicate, the plasm being held together by bulky fats, some of them possibly in a fluid state, a certain proportion of which exist as fatty acids soluble in water or as compounds easily hydrolyzed.

All these factors contribute to the rapid imbibition of water. With the edema of the cells, or following it, there occurs an outpouring into the fluid of fats, of compounds containing phosphorus, and of nitrogen as proteid or proteid split products, and as ammonia. The occurrence of volatile fatty acids and amino nitrogen in fresh autolysates suggests a possible source in a leucin-like substance, one that is associated with advanced proteolysis. This assumption would imply either that hydrolysis is extremely rapid or that considerable amounts of such substance exist preformed in the cocci or about them. The evidence points to the immediate liberation by excess of water of many products representing past enzyme activity.

6. Osterhout: Science, 1915, 41, p. 255.

The process of lysis, as considered in this paper, is complete within a few minutes and constitutes a primary stage in the disintegration of the organism. This may be hastened by shaking and by centrifugalization. The substances liberated into the fluid now undergo fairly rapid qualitative and quantitative changes, but certain proportions of nitrogen and fat persist in one or another form for long periods. At the conclusion of this stage the substance of the cocci contains residual proteid which is not liberated at once by fresh fluid but appears to be somewhat slowly converted within the remnants of the cells into simpler form. There is, then, no constant increase of nitrogen in the fluid.

The existence of enzymes in the live cocci is well known. One is proteolytic, acting best in a slightly alkaline medium and destroyed at 56 C.; a second is fermentative, splitting dextrose, while a third splits neutral fat and does not, so far as I have been able to determine, possess reversible action. The activity of the latter appears to cease at 55 C. It will be seen from the foregoing tables that lysis occurs at all temperatures, even that at which proteid is coagulated and at points where enzyme activity is destroyed or at least checked. The toxicity of autolysates for animals is not checked at 58 C., but the temperature apparently hastens the lysis and carries the substances beyond the toxic stage more quickly than temperatures of 37 C. or 20 C.

The influence of the character of lysis on immunity will be discussed in a later paper.

CONCLUSIONS

Lysis of the gonococcus in water and in salt solution is probably due, not to the activity of enzymes, but to other causes, among which water permeability and solution of fatty substances play important parts.

Lysis is probably initiated by excess of water.

The gonococcus is capable of retaining viability for considerable periods of time in more or less anhydrous substances such as glycerol, lanolin, vaselin, and cold cream.